



Dietary wheat amylase trypsin inhibitors exacerbate murine allergic airway inflammation

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Abstract

Background Wheat amylase trypsin inhibitors (ATI) are dietary non-gluten proteins that activate the toll-like receptor 4 on myeloid cells, promoting intestinal inflammation.

Aim of the study We investigated the effects of dietary ATI on experimental allergic airway inflammation.

Methods Mice on a gluten and ATI-free diet (GAFD), sensitized with PBS or ovalbumin (OVA) and challenged with OVA, were compared to mice on a commercial standard chow, a gluten diet naturally containing ~0.75% of protein as ATI (G+AD), a gluten diet containing ~0.19% of protein as ATI (G-AD) and a GAFD with 1% of protein as ATI (AD). Airway hyper-reactivity (AHR), inflammation in bronchoalveolar lavage (BAL) and pulmonary tissue sections were analyzed. Allergic sensitization was assessed ex vivo via proliferation of OVA-stimulated splenocytes.

Results Mice on a GAFD sensitized with PBS did not develop AHR after local provocation with methacholine. Mice on a GAFD or on a G-AD and sensitized with OVA developed milder AHR compared to mice fed a G+AD or an AD. The increased AHR was paralleled by increased BAL eosinophils, IL-5 and IL-13 production, and an enhanced ex vivo splenocyte activation in the ATI-fed groups.

Conclusions Dietary ATI enhance allergic airway inflammation in OVA-challenged mice, while an ATI-free or ATI-reduced diet has a protective effect on AHR. Nutritional wheat ATI, activators of intestinal myeloid cells, may be clinically relevant adjuvants to allergic airway inflammation.

Keywords Amylase trypsin inhibitors · Gluten · Wheat sensitivity · Innate immunity · Allergic airway inflammation

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Abbreviations

AAI	Allergic airway inflammation
AHR	Airway hyper-reactivity
ATI	Amylase trypsin inhibitor
BAL	Bronchoalveolar lavage
G	Gluten
GFD	Gluten-free diet
HLA	Human lymphocyte antigen
IL	Interleukin
NCWS	Non-celiac (non-allergy) wheat sensitivity
OVA	Ovalbumin
PAS	Periodic acid–Schiff
TLR	Toll-like receptor

Introduction

Diet is an important environmental factor that modulates the development of allergic airway inflammation [1, 2], pointing towards a direct and indirect effect of nutritional components on immune cells. Although the exact mechanism by which nutrition affects immune responses in the lungs remains unclear, the association between the respiratory and gastrointestinal systems (the gut–lung axis) seems to be strongly influenced by dietary modifications.

Dietary changes such as an increased consumption of fiber and their products of fermentation, for instance, short chain fatty acids can attenuate allergic inflammatory responses in mice [1], reduce the incidence of asthma in humans [2] and an early exposure to unpasteurized milk correlated with a lower incidence of asthma and allergies during childhood [3]. However, dietary components can also worsen inflammatory conditions, as exemplified by wheat consumption worsening intestinal inflammatory diseases [4–6] or dietary fat worsening diabetes [7].

Wheat has been traditionally consumed as part of a balanced diet in most Western countries. However, around 1% of the population of these countries is affected by celiac disease, a Th1 T cell-mediated immune reaction to certain gluten peptides, which results in partial destruction of the proximal small intestinal mucosal architecture. A larger proportion of people is affected by non-celiac wheat sensitivity (NCWS), a reaction to various components of wheat, which either manifests as abdominal pain, diarrhea or bloating, or with a broad range of extraintestinal symptoms [6, 8, 9]. Here NCWS encompasses IgE-negative wheat allergies [10], possibly sensitivity to fermentable oligo-, di- and monosaccharides and polyols (FODMAPs) which are relatively high in wheat, and the potential disease-promoting activity of wheat amylase trypsin inhibitors (ATI) [4, 11–13].

Wheat amylase trypsin inhibitors (ATI) are non-gluten wheat proteins, largely resistant to intestinal proteolysis and with the capacity to trigger innate inflammation by engaging the toll-like receptor 4 (TLR4)-MD2-CD14 complex on dendritic cells, macrophages and monocytes, leading to the release of pro-inflammatory cytokines and chemokines and an up-regulation of dendritic cell maturation markers [4].

Notably, this intestinal stimulation does not only lead to worsening of low-level pre-existing small intestinal and colonic inflammation, as occurs in a model of inflammatory bowel disease [9], but the inflammatory signal is propagated to the mesenteric lymph nodes where intestinal as well as extraintestinal T cells undergo antigen priming [12]. Thus, consumption of wheat ATI could be an important dietary factor that dose-dependently contributes

to the promotion of extraintestinal diseases [5, 6, 12, 14]. To test this hypothesis, we evaluated the effects of defined amounts of dietary ATI on exacerbation of experimental allergic airway inflammation, using a well-characterized murine model of OVA-induced airway sensitization.

Materials and methods

Diet preparation

Customized murine pellets were prepared based on a gluten and ATI-free diet (GAFD) (Ssniff, Soest, Germany) with a defined carbohydrate content and casein as main protein source as described by us before [12]. In addition, three diet preparations containing different protein amounts of gluten and ATI were manufactured, namely: diet 1, 25% gluten, naturally containing ~0.75% ATI (G+AD); diet 2, 25% gluten de-enriched of ATIs, after repeated extractions with salt solutions, the amount of ATI was ~0.19% (G–AD), and diet 3, a GAFD containing 1% of protein as purified ATI (AD). Purified ATI and gluten de-enriched of ATI were prepared as described by us [12]. Briefly, for de-enrichment gluten was extracted three times using 50 mM ammonium bicarbonate (Abic) buffer, dialyzed, sterile filtrated and lyophilized. ATI extracted with the same buffer were isolated by fractional salt precipitation using ammonium sulfate. Further purification of ATI by FPLC was used only for in vitro experiments.

ATI bioactivity assays of enzymatically digested food pellets

Pellets were enzymatically digested and ATI bioactivity measured as described before [12]. Briefly, 1 g of milled, sieved and defatted samples were subjected to pepsin (P700, Sigma-Aldrich, Munich, Germany; 10 mg in 10 ml 0.1 M HCl, pH 1.8) at 37 °C for 2–4 h, adjusted to pH 7.8 with NaOH and incubated with trypsin (T9201, Sigma-Aldrich; 10 mg in 0.5 ml PBS) at 37 °C for 2–4 h. The pH was adjusted to 4.5 with HCl and N α -tosyl-L-lysine chloromethyl ketone hydrochloride [90182, Sigma-Aldrich (10 mg in 0.5 ml DMSO)] was added to block residual trypsin. Samples were then centrifuged at 2500 $\times$ g for 20 min and the supernatant was dialysed against 10 mMol ammonium bicarbonate, pH 7.8 at 4 °C, sterile filtrated and freeze-dried. The dried material was reconstituted in PBS or other cell compatible media and added to THP-1 monocytes–macrophages (TIB-202, ATCC, Wesel, Germany) at a concentration of 10–250 μ g protein/ml of ATI extracts and incubated overnight. Release of the innate cytokine IL-8 into the medium was quantified with a validated ELISA (BD, Heidelberg; eBioscience, Frankfurt, Germany; R&D systems, Minneapolis, USA).

Mice

Female C57BL/6 mice, age 8–12 weeks, were purchased from Harlan (An Venray, the Netherlands), raised on a standard chow (SC) diet (Ssniff, Soest, Germany) and then placed on a defined GAFD for at least 4 weeks before starting the experiment. Mice were then divided in five groups: group 1, continued on a GAFD and mock-sensitized with PBS; group 2, continued on a GAFD; group 3, changed to a G+AD (containing amounts of ATI equivalent to the human wheat based diet [12]); group 4, changed to a G–AD; and group 5, changed to a AD. Groups 2–5 were sensitized with OVA and all groups (1–5) were challenged with OVA, as described before [15, 16] with minor modifications. Briefly, mice were sensitized subcutaneously with 10 µg/ml OVA (Calbiochem, LaJolla, CA, USA) or phosphate-buffered saline (PBS) once per week during 3 weeks, then each group of mice was challenged with OVA (10 mg/ml) three times every 48 h for 30 min using a nebulizer. All experiments were approved by the Animal Care and Use Committee of the state of Rhineland Palatinate, Germany.

Lung function and collection of bronchoalveolar lavage

Airway hyperresponsiveness (AHR) was measured invasively in anesthetized mice by administration of increasing doses (0–100 mg/ml) of methacholine (MCh) using a body plethysmograph (Buxco Electronics, Inc., Wilmington, NC, USA) as described before [16, 17]. Data are reported as airway resistance (RI) above the baseline value taken before receiving MCh. After measurement of AHR, lungs were lavaged by gentle flushing with 1 ml PBS supplemented with 10% FCS. Bronchoalveolar lavage (BAL) was recovered and centrifuged. The supernatant was collected and immediately frozen at -20°C until determination of cytokines and the cell pellet was used for cytospin preparations.

Cytospin preparation of BAL cells

BAL cells were prepared and stained as described before [16]. Briefly, the BAL pellet was resuspended and live cells were counted by means of trypan blue exclusion. Approximately 5×10^5 cells were washed, resuspended in 100 µl PBS and centrifuged (Shandon cytospin centrifuge, Thermo Electron, Langenselbold, Germany) onto a glass slide for 5 min at 500 rpm, followed by air drying and staining with a differential quick stain kit (modified Giemsa, Polysciences, Hirschberg, Germany). Cell assessment was performed on at least 300 cells per slide based on morphology and staining behavior using a microscope with digital camera (AxioCam MRC camera, Carl Zeiss, Oberkochen, Germany). The absolute cell number (cell/ml BAL) was obtained after

multiplying the percentage of cells with the total cell number of each cell population (macrophages, neutrophils, eosinophils and lymphocytes).

Proliferation assay

Ex vivo stimulation of splenocytes was performed as described by us [16]. Briefly, $1\text{--}5 \times 10^6$ cells/well from each mouse were cultured on 96-well flat-bottom microtiter plate (CorningCostar) in a volume of 0.2–1 ml culture medium (Iscove's Modified Dulbecco's, Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS; PAN Systems Nürnberg, Germany), 50 µM 2-mercaptoethanol (Carl Roth GmbH & Co., Karlsruhe, Germany), 2 mM l-glutamine and 100 U/ml penicillin/100 mg/ml streptomycin (Gibco) alone or with various concentrations of OVA (1–100 µg/ml) as indicated. After 72 h, ^3H -thymidine (1 µCi/well) was added to the cultures for another 16–18 h. Levels of incorporated ^3H -thymidine were determined by liquid scintillation counting.

Cytokine levels

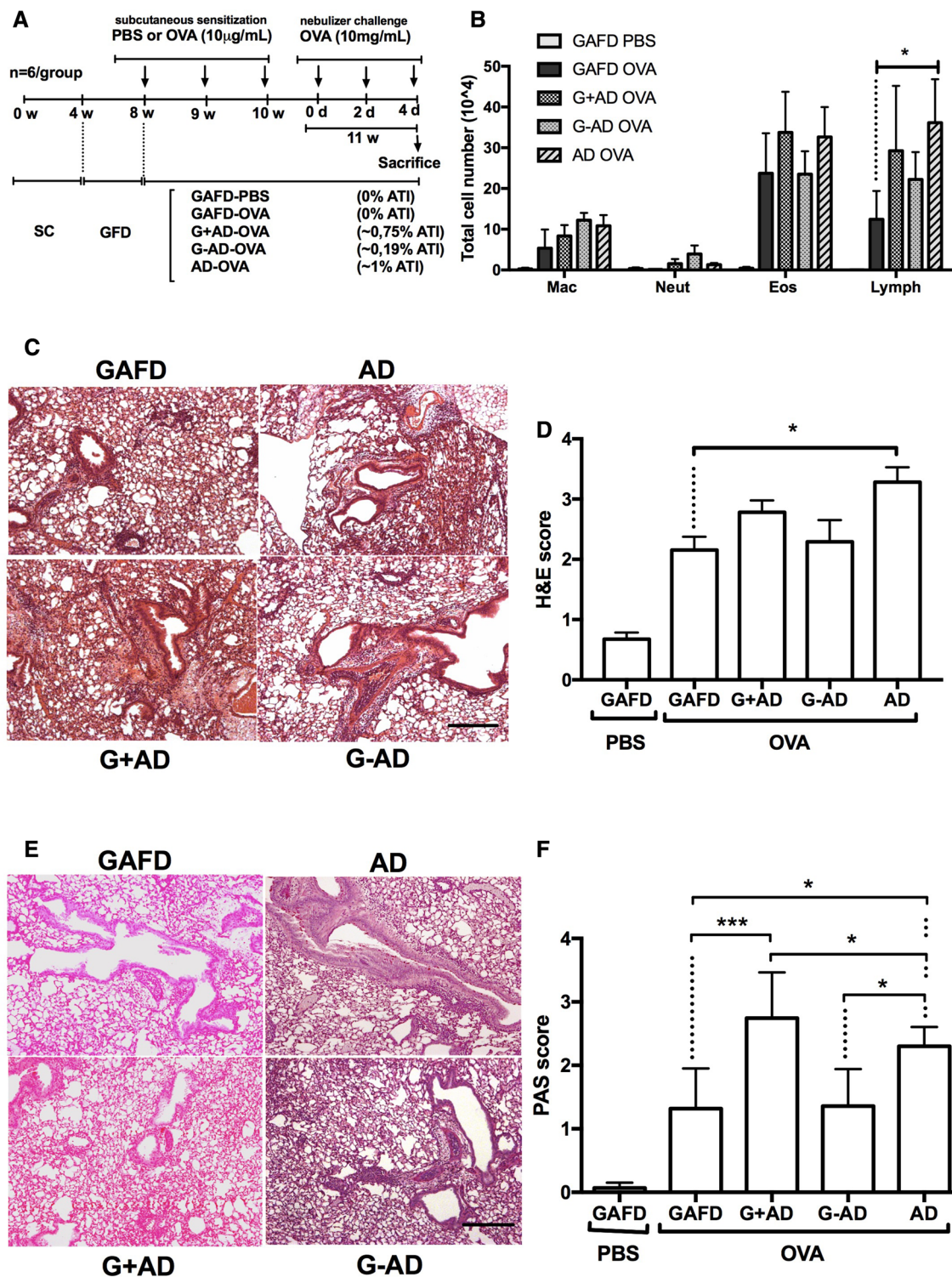
Determination of cytokine (IL-5, IL-13) levels in BAL and supernatants from stimulated splenocytes from all groups were measured by validated sandwich ELISAs (eBioscience, Frankfurt, Germany; R&D Systems, Wiesbaden, Germany), according to the manufacturer's instructions.

Lung histology

Lungs were prepared for histological assessment as described by us and others [16, 18]. Briefly, lungs were perfused and filled with 4.5% formaldehyde (Roth, Karlsruhe, Germany), fixed for 24 h before dehydration and paraffin embedded. Then, 1 µm lung sections were stained with hematoxylin/eosin (H&E) and periodic acid–Schiff (PAS). The degree of inflammatory cell infiltration and the proportion of activated goblet cells indicative of mucus production were evaluated using a microscope (Zeiss Axio Scope A.1), an AxioCam MRC camera (Carl Zeiss, Oberkochen, Germany) and scored applying a scale [18] from 0 (no cell infiltration, no activated goblet cells) to 4 (severe peri-bronchial cellular infiltration at multiple sites, 75–100% activated mucus-producing goblet cells).

Statistical analyses

Data was statistically analyzed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA). Multiple comparisons were performed by one-way or two-way analysis of variance. Differences among selected experimental groups with P values < 0.05 were considered significant.



Results

Dietary ATI promotes infiltration of inflammatory cell in the lungs

To test the hypothesis that dietary wheat proteins influence

allergic airway inflammation (AAI), we used defined quantities of gluten and ATI. Thus, mice raised on a SC diet were placed on a GAFD for at least 4 weeks and then either continued on the GAFD or changed to: G+AD, G-AD and AD, respectively. At the same time, mice on a GAFD were sensitized with either PBS (control) or OVA and subsequently

Fig. 1 ATI consumption promotes infiltration of inflammatory cells in the lungs. **a** Experimental protocol, C57BL/6 mice ($n=6$) were raised on SC diet, placed on the GAFD for 4 weeks and divided in five groups: group 1, continued on the GAFD and mock-sensitized three times with PBS; group 2, continued on the GAFD; group 3, changed to a diet containing 25% gluten and ~0.75% ATI (G+AD); group 4, changed to a diet containing 25% gluten and ~0.19% ATI (G-AD); and group 5, changed to a diet containing 1% purified ATI (AD). Groups 2–5 were sensitized three times with OVA and all groups (1–5) were challenged three times with OVA. **b** Differential cell counts in the BAL. *Mac* macrophages; neut, neutrophils, *eos* eosinophils; lymph, lymphocytes. **c** Representative H&E-stained lung tissue from mice in all groups. Scale bars, 200 μ m. **d** H&E score of all groups. **e** Representative PAS-stained lung tissue from all groups. Scale bars 200 μ m. **f** PAS score of all groups. All results are representative of three different experiments and are expressed as the mean $\pm$ SEM ($n=6$ per group). Statistical significance was determined with one- or two-way analysis of variance (ANOVA). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

challenged with OVA. Mice on other dietary regimes, namely G+AD, G-AD and AD were sensitized and challenged with OVA (Fig. 1a).

There was a significant increase of inflammatory cell subsets in the BAL from all groups challenged with OVA compared to mice on the GAFD challenged with PBS (controls) and we observed a non-significant attenuation of inflammatory cell infiltration in the OVA-challenged mice on a GAFD compared to all other groups consuming ATI, either alone or combined with gluten (Fig. 1b). Furthermore, mice sensitized and challenged with OVA that consumed a G+AD and AD showed enhanced histological inflammatory scores [18], as demonstrated by an increased peri-bronchial inflammatory cell infiltration at multiple sites of H&E-stained lung sections (Fig. 1c, d). This observation was paralleled by PAS-stained lung sections, examining goblet cell metaplasia, here was noted that mice on G-AD had significantly lower scores of goblet cell hyperplasia that were comparable to mice on a GAFD (Fig. 1e, f).

In addition, we tested the effect of a SC diet (containing bioactive ATI) against a GAFD in mice sensitized and challenged with OVA or PBS. We found that, although not reaching significance, inflammatory cell infiltration was lower in the mice consuming a GAFD, as shown by a reduction in eosinophilic and lymphocytic infiltrates, reduction histological scores of H&E- and PAS-stained lung sections compared to mice on SC diet. In line with this observation, attenuated AHR and lower concentration of BAL IL-13 indicated that our SC contains enough bioactive ATI to trigger AAI (Suppl. Figures 1 and 2).

Biologically active ATI diet enhances airway inflammation

To ensure that the TLR4 stimulating activity of ATI was still present in the food pellets, we extracted ATI under

conditions of intestinal digestion and evaluated the biological activity of all dietary preparations by exposing extracts to human THP-1 monocytes/macrophages and measure release of IL-8 (Fig. 2a). Thus, a G+AD and an AD had three–four-fold higher ATI activity than a GAFD, in line with previous observations on ATI stability under digestive conditions. As outlined by us before, when calculating the pharmacological equivalence dose [9], a content of 1% of the dietary protein as ATI is comparable to the ATI present in a normal human diet that contains ~150 g of wheat per day. Furthermore, after local provocation with increasing doses of aerosolized methacholine, a non-selective muscarinic receptor leading to bronchoconstriction, AHR was measured as airway resistance (RI), indicating that mice on a G+AD and AD had enhanced AHR compared to mice on a G-AD and mice on GAFD (Fig. 2b, c). This finding was paralleled by increased BAL levels of IL-5 and IL-13 (Fig. 2d).

ATI consumption stimulates splenocytes release of Th2 cytokines

The proliferation rate of splenocytes from OVA-immunized mice after ex vivo restimulation with different concentrations of OVA was consistently higher in the groups consuming ATI, either alone or combined with gluten, compared to the control GAFD (Fig. 3a), especially after stimulation with lower OVA concentrations (Fig. 3b). Moreover, secretion of key Th2 cytokines (IL-5 and IL-13) from splenocyte cultures followed the same pattern (Fig. 3c, d).

Discussion

Wheat has become one the main dietary staple around the world and its protein consumption has been linked to the development of celiac disease, wheat allergy and non-celiac wheat sensitivity (NCWS), the latter being characterized by a multifaceted clinical presentation with intestinal and extraintestinal symptoms due to innate immune activation [5, 6]. We recently showed that the TLR4-stimulating properties of dietary ATI enhance pre-existing intestinal inflammation characterized by disruption of intestinal homeostasis and barrier function with increased numbers of activated myeloid cells (mostly macrophages and dendritic cells), which after activation migrate to the mesenteric lymph nodes (MLN) rather than remain in the lamina propria where they could prime T cells that circulate to the site of inflammation [12]. In the present study, our main finding is that consumption of wheat ATI worsens an extraintestinal inflammatory condition, namely murine allergen induced airway inflammation (AAI).

We found that consumption of ATI, either alone or combined with gluten during 3 weeks was able to increase

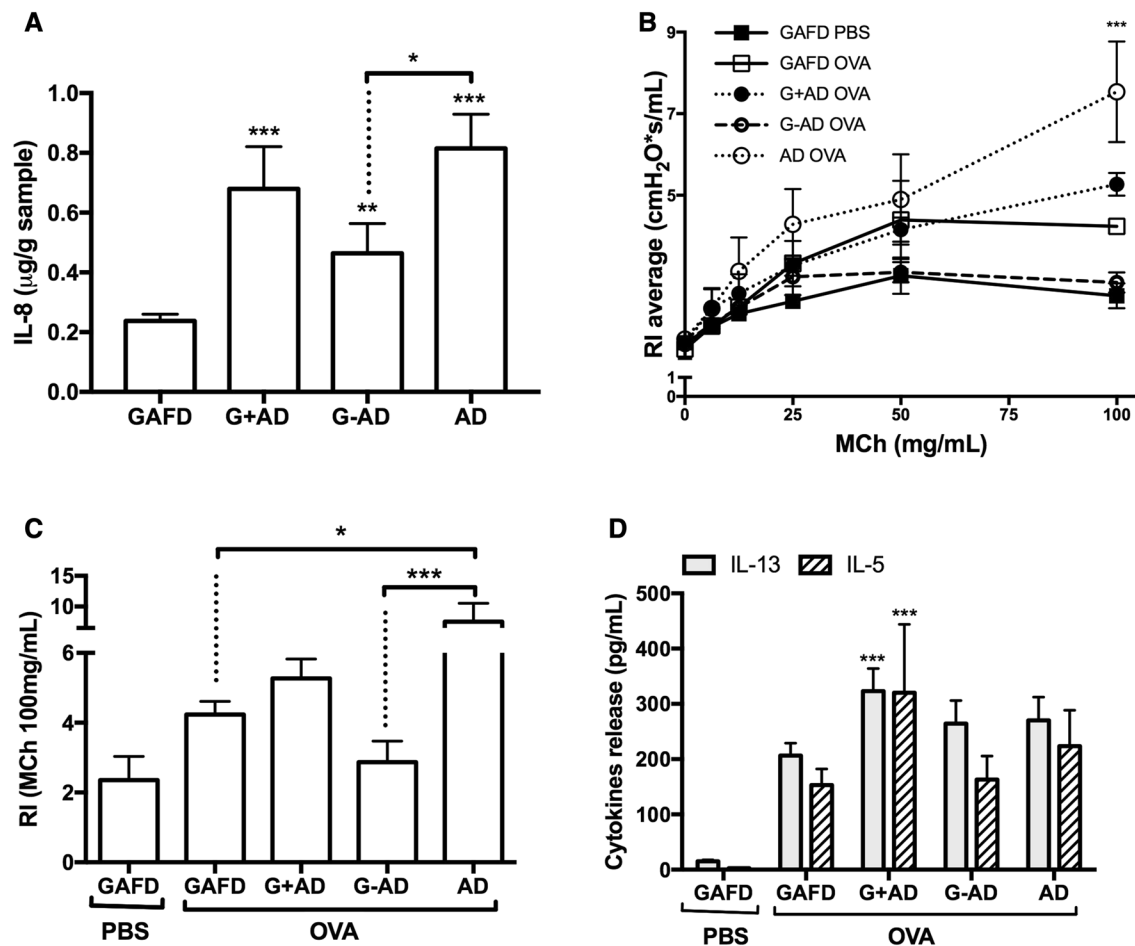


Fig. 2 Biologically active ATI diet enhances airway inflammation. **a** Bioactivity assay of all dietary pellets expressed as THP-1 cell IL-8 release in µg/g of sample. **b** Measurement of AHR, as assessed by airway resistance to increasing doses of methacholine in all groups. **c** Measurement of AHR, as assessed by 100 mg/ml methacholine.

d Concentration of IL-5 and IL-13 levels in BAL. All results are representative of three different experiments and are expressed as the mean ± SEM ($n=6$ per group). Statistical significance was determined with one- or two-way analysis of variance (ANOVA). * $p<0.05$, *** $p<0.001$

infiltration of eosinophils and lymphocytes in the lungs of mice sensitized and challenged with OVA. This cell infiltration produced enhanced levels of inflammatory Th2 cytokines (IL-5 and IL-13), combined with histological evidence of abundant mucus-producing goblet cells and elevated AHR after methacholine challenge. Moreover, isolated splenocytes from those mice were restimulated with exogenous OVA to trigger activation of MHC-II restricted OVA-reactive CD4⁺ T cells which hyperproliferated in mice consuming ATI, this was mirrored with an upregulated Th2 immune response reflected by an increased release of IL-5 and IL-13. These effects were not observed on mice consuming a diet de-enriched of ATI (G-AD) with lower number of BAL eosinophils and lymphocytes, reduced BAL cytokines (IL-5 and IL-13), less mucus-producing goblet cells and an attenuated AHR that was comparable to mice on a GAFD. Thus, suggesting that wheat ATI rather than gluten proteins are responsible for the worsening of AAI.

Recruitment of inflammatory cells to the airways is orchestrated not only by adaptive but also by innate immune mechanisms, with innate immune signals serving as adjuvants to the adaptive response [19, 20]. Here, we can demonstrate that a defined nutritional trigger, i.e., ATI from wheat, initiate activating innate immune signals in the gut, triggered via TLR4 of myeloid cells that finally reach the lungs to promote an ongoing adaptive immune response (AAI). Since we could not detect ATI in the blood of mice fed ATI (data not shown), their promotion of AAI likely occurs via myeloid cells activated in the intestine that migrate towards the periphery, as shown by us in a mouse model of gut inflammation [12]. How far this would also translate to nutritional allergies, with their intestinal and, e.g., skin and pulmonary manifestations, needs to be shown, but a similar adjuvant effect of ATI in nutritional allergies is likely.

Thus, in a study looking at the combined effects of food and respiratory allergens, the authors used gliadin (gluten

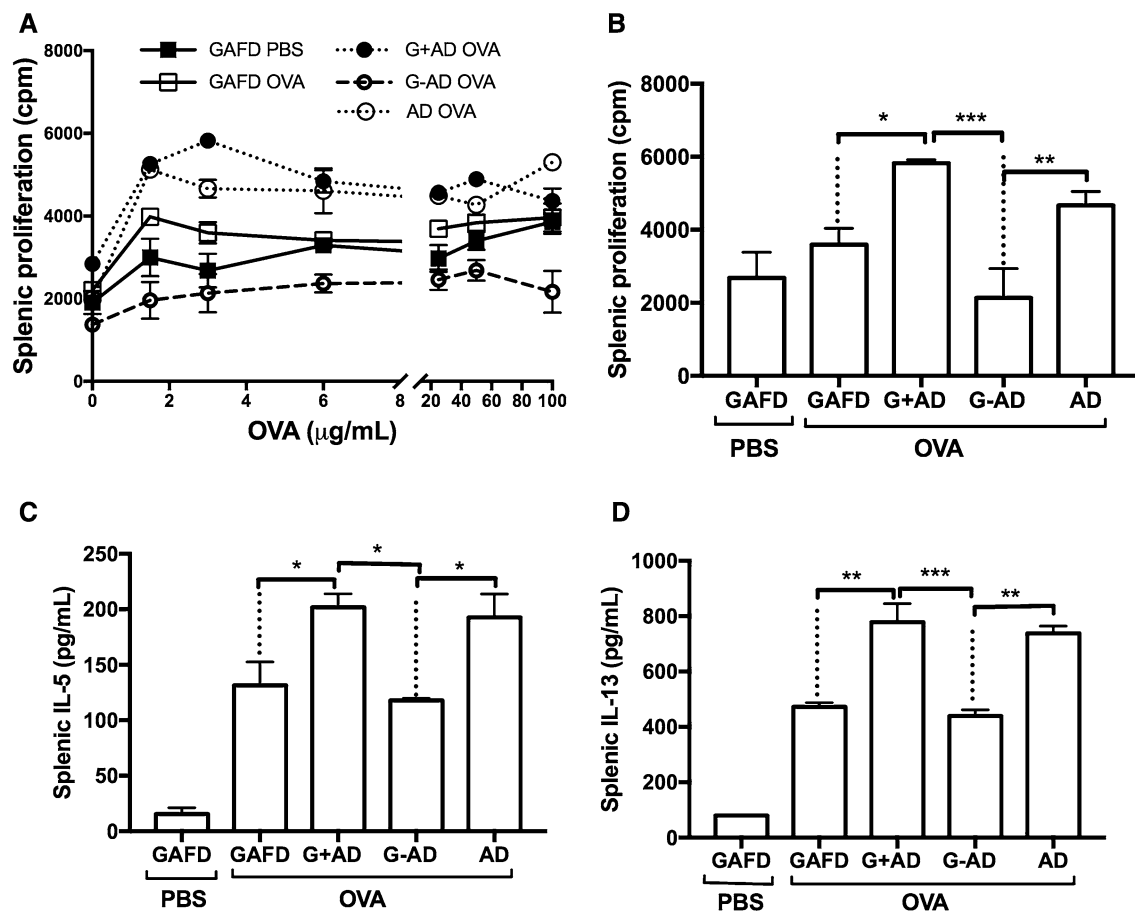


Fig. 3 ATI consumption stimulates splenocytes release of Th2 cytokines. **a** Measurement of splenocyte proliferation after increasing doses of OVA. **b** Proliferation of splenocytes after challenge with 1.5 μg/ml OVA. **c**, **d** IL-5 and IL-13 levels in splenocyte superna-

tants. All results are representative of three different experiments and are expressed as the mean $\pm$ SEM ($n=6$ for all groups). Statistical significance was determined with one- or two-way analysis of variance (ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

proteins) to sensitize and orally challenge mice that were subsequently exposed to a respiratory allergen (house dust mite); they observed enhanced intestinal but not lung inflammation [21]. This suggests that allergic sensitization via the lungs can also take the reverse route, i.e., affect intestinal inflammation to a target antigen. However, there was little information about the dietary regime of these mice and whether wheat proteins were used as ingredient for manufacturing mouse feed.

When we analyzed a standard chow diet, we found that it contained enough amounts of bioactive ATI that induced a modest but noticeable AAI response when compared to a GAFD. Unfortunately, the exact amount and source of ingredients in closed formula pellets are often defined with broad terms such as “vegetable proteins”, suggesting the use of a wide range of raw ingredients that change according to seasonal and market availability [22]. While this should not affect the general dietary requirements of mice, in an experimental setting and considering that wheat ATI promote inflammation, standard chows with a variable content

of wheat or wheat proteins could partly explain the lab-to-lab variability observed in certain murine models [23].

To conclude, we provided evidence that consumption of nutritional wheat ATI contributes to the severity of AAI, an extraintestinal inflammation previously thought to be localized exclusively to the lungs. Additionally, we observed that gluten de-enriched of ATI, had no significant effect on AAI. Whilst further studies are needed to determine the exact pathomechanism of lung inflammation promoted by dietary ATI, a central role of ATI-activated, migratory myeloid cells appear likely. Our study opens the possibility of clinical studies in which a dietary treatment consisting of elimination or reduction of ATI could positively impact the management of AAI.

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Author contributions VZ: study design; acquisition of data; analysis and interpretation of data; drafting and editing of the manuscript;

statistical analysis. VR: acquisition of data and support during experiment and data acquisition/analysis (stimulation of splenocytes). JM and PS: acquisition of data and support during in-vivo experiments (lung measurements). KS and other authors: support in revising the manuscript. DS: study design; interpretation of data; study supervision; drafting and editing of the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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